PTK00316

Cultured Neuron Probe

Contract No. N01-NS-3-2393

Quarterly Report No. 10

July 1, 1995 - September 30, 1995

California Institute of Technology

Michael Maher

Jerome Pine

Steven Potter

Yu-Chong Tai

John Wright

Rutgers University

Anatol Bragin

Gyorgi Buzsaki

This QPR is being sent to you before it has been reviewed by the staff of the Neural Prosthesis Program

General Introduction

Our aim is to develop a cultured neuron probe. This consists of a silicon structure into which individual dissociated neurons can be placed, and which can be inserted into an intact nervous system. Furthermore, within the structure each neuron is in close proximity to an electrode, by means of which it can be stimulated, or its activity can be recorded, through electrical leads which connect to external electronics. It is hoped that neurons in the probe will synaptically integrate with the host nervous system, to provide a highly specific, relatively non-invasive, two-way communication channel. If this occurs, the methodology has important possibilities for neural prostheses. The goal of this project is to perform initial experiments to establish the feasibility of communication by means of a neuron probe. The tissue we have chosen in which to initially implant the probe is the rat hippocampus. If initial studies are successful, probes will be designed and implanted for communication with sensorimotor cortex.

The neuron probe we plan to fabricate will be made of micromachined silicon and will have sixteen electrodes: one conventional electrode to detect activity during placement of the probe, and fifteen within wells into which neurons will be placed. Its configuration will be similar to that of passive multielectrode probes which have been developed. It will be implanted when the cultured neurons are very young, and after a time of weeks it is hoped that they will have survived and made two-way synaptic connections. By stimulation and recording in the host and probe, we will test for the existence of such connections. An essential feature of the experiments is that the viability of the implanted neurons will be independently determined over time by stimulating them and recording their resulting action potentials.

Summary

During this quarter, a variety of different "tunnel" wells were fabricated and tested. These wells have outgrowth paths through tunnels rather than through openings in the grillwork. Tunnels were all 15 microns long, and varied in cross section from 0.5 x 10 microns to 0.2 x 2 microns. In all cases neuronal outgrowth appeared unconstrained. For over 200 neurons in culture for 6 days or more, there were three escapes, all from 10 micron wide wells. It seems clear that a well design has finally been created which solves the escape problem and promotes good outgrowth. In addition, the well design provides a canopy under which processes grow, which can protect initial outgrowth during insertion into cortex.

Based on the tests, it was decided to make large tunnels, 4 and 10 microns x 0.5 microns high, since escape through them was not serious and the large size offered the least chance of inhibiting growth at later stages of cell development. Production of new dummy probes and neurochips with electrodes was begun. In addition, a protective canopy extending 30 microns from the well will be used for probes, to maximize the protection of the proximal dendrite and axon outgrowth.

A second *in vitro* project has been the development of the hippocampal slice culture system, to study neural outgrowth into the slice as a model for the outgrowth *in vivo*. Neural staining has been further perfected to make possible observation of the neurons growing into the slice. And the use of two-photon microscopy has been refined, to permit recording of time lapse images of neural outgrowth with 100 exposures or more during times of order one day. An initial test with three probes inverted over slices appeared to stop growth from all the neurons. For comparison, identically stained neurons were deposited on cultured slice surfaces and grew beautifully. Both cell bodies and processes migrated into the slice, with some processes extending sompletely through the slice, over 100 microns in thickness. Studies of this rather mysterious phenomenon will be pursued during the coming quarter.

Experiments using probes implanted in rats have been made in which micro transplants have also been made for comparison. Transplant outgrowth was seen after periods of weeks, but no probe outgrowth. As a next step, acute probe experiments were done in which probes were inserted into an anesthetized rat and then removed after ten minutes to two hours. No outgrowth was seen for a total of three probes. This mirrors the phenomenon observed in slices, although the environment is certainly not identical and the problems may turn out to be unrelated. In any case, intensive efforts will be made in the coming quarter to ascertain whether the cause is cell death, and if so as a result of what environmental stress.

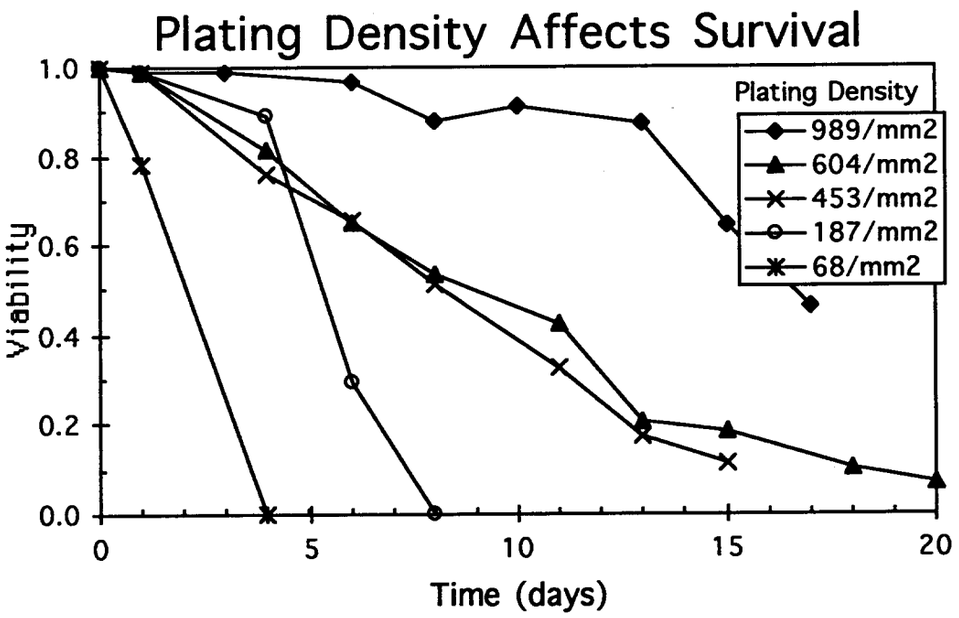
Growth of hippocampal neurons in chips with canopy grillwork

We have successfully achieved growth of hippocampal neurons from the dummy chips with canopy grillwork. We have concentrated mainly on the straight, 15 μm wide canopies. The 30 μm wide canopies have too little room between the wells for good imaging of growing processes on either chips or probes. For the wide canopies it was nearly impossible to determine the origins of processes, and hence the number of growing cells, once the cells had been in culture more than a few days.

Neuron Survival in Mass Cultures

We have been working on methods to increase the longevity of dissociated cell cultures. Long-lived cultures with good survival are important for assuring that escape does not take place even over long periods. Neurobasal medium (NB/B27) was optimized by iots developers for maximal survival at 4 days in culture, but our neurons typically need two weeks to mature. In our hands, cell survival is typically over 80% at four days, then falls linearly to zero over a period of 4 days to 3 weeks afterwards (see the figure below). This experience is typical among other workers in the field, as confirmed by Paul Price at Gibco, and is insufficient for our needs.

The graph below demonstrates that the longevity of the cultures in standard NB/B27 medium is dependent upon plating density, with higher density yielding better survival. Morphology of dead and dying cells in these

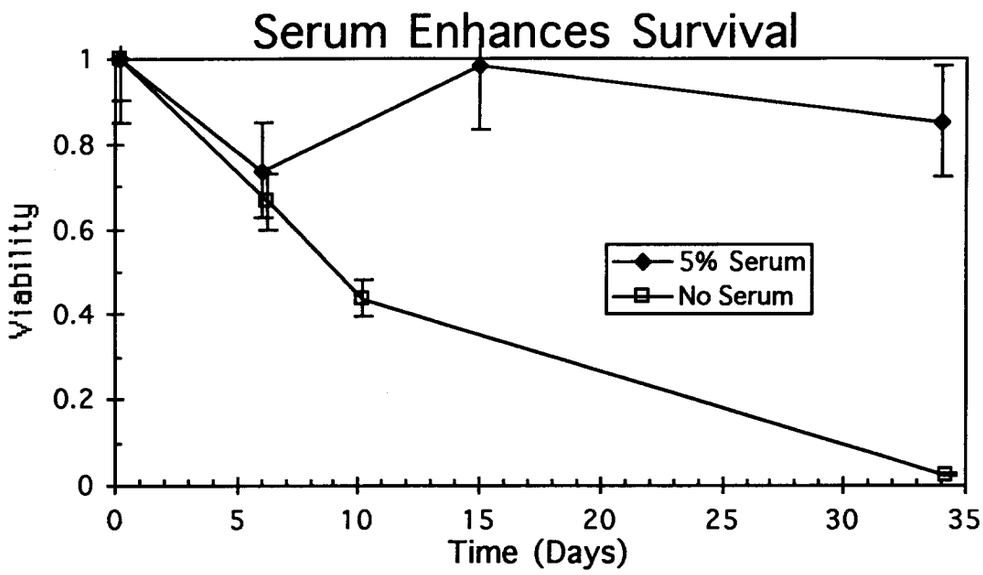


cultures is consistent with apoptosis (blebbing on cell bodies and processes, fragmentation of the nucleus), and a DNA electrophoresis gel of a standard 3-week old culture exhibited a low-molecular weight pattern also consistent with the DNA fragmentation associated with apoptosis.

Neurobasal supports survival of glia, but lacks some factor necessary for cell division. Under normal culture conditions, less than 5% of the cells from E18 rat hippocampi are glia. Before Neurobasal was developed, hippocampal neurons were typically grown by Banker for many weeks by co-culturing with glia in defined serum-free medium. It is possible that glia normally produce a factor which inhibits apoptosis in the neurons, and that higher plating densities provide enough glia to raise the concentration of this factor high enough to support the neurons.

To test this possibility, we encouraged glial growth in some cultures by adding 5% defined horse serum (Hyclone) to the NB/B27 medium. Glia rapidly proliferate, and the glial population exceeds the neuronal population within 3 days. Glial confluence occurs after 10-14 days in culture, depending upon the initial plating density. Neuronal survival with serum after 34 days in culture was high, and far better than the serum-free control, as indicated in the table and figure below. These results suggest that the survival problem could easily be solved by adding serum, if the presence of glia does not affect the functioning of the neurochips and the observation of neurons in wells.

Medium	Survival at 34 DIC	Plating Density	Number of Cultures
NB/B27 + 5% serum	86%±7%	1320±260/mm^2	2
NB/B27	2.3%±0.2%	1544±290/mm^2	2



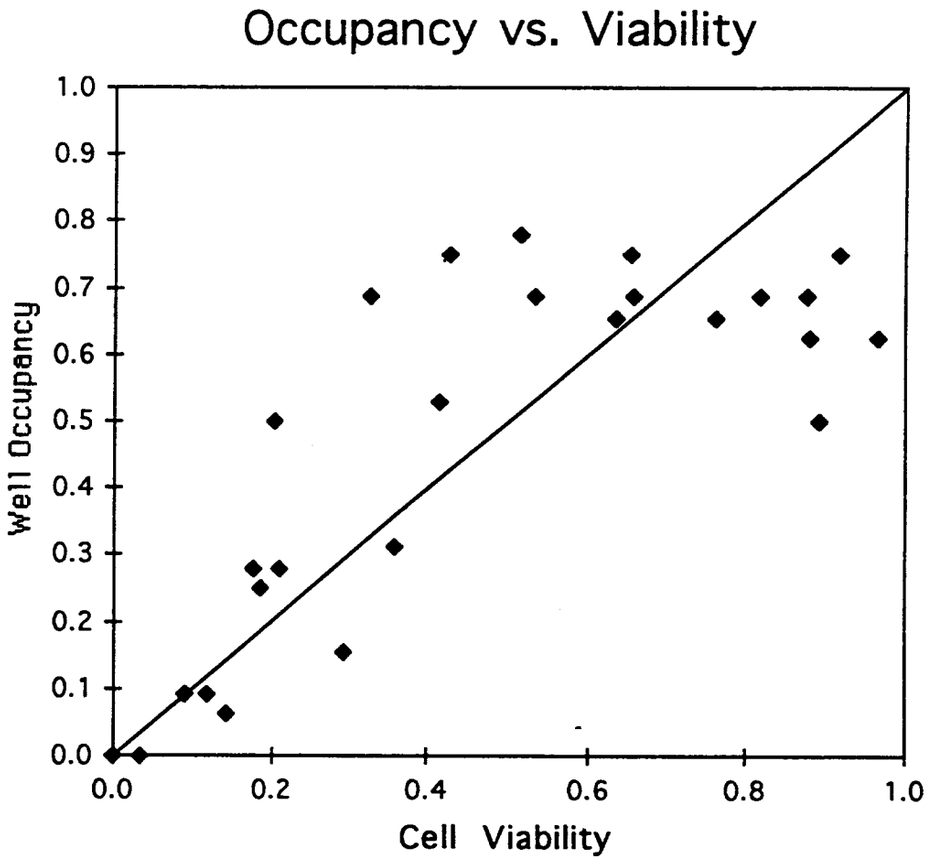
Survival in Wells

Two patterns of sixteen wells each on thirty-two chips were loaded with embryonic rat hippocampal neurons, using the standard serum-free NB/B27 medium. Of these 32, 12 chips had surviving neurons after six days in culture. Ten chips accidentally succumbed to ethanol toxicity, due to improper technique. Tools were sterilized with ethanol, and were not completely dried before being placed into the medium. Enough ethanol could be transferred by multiple tools to bring the concentration above the toxic threshold of 1%. We have since begun to sterilize tools using heat, and the toxicity problem disappeared. Five cultures died before 6 days due to infection. The cause of early death in the remaining six cultures is not known, but some could possibly be attributed to apoptosis. Discounting the avoidable ethanol toxicity, we have a 55% success rate at keeping cells alive for 6 days. On these 12 living chips, 209 wells had living cells at 6 days in culture, for an average of 55% probability that a cell loaded into a well will survive.

To determine whether or not being loaded into a well is harmful to the cell, we measured the viability rate of cells growing elsewhere on the chip, away from the well region on flat silicon, concurrently with observations of cells

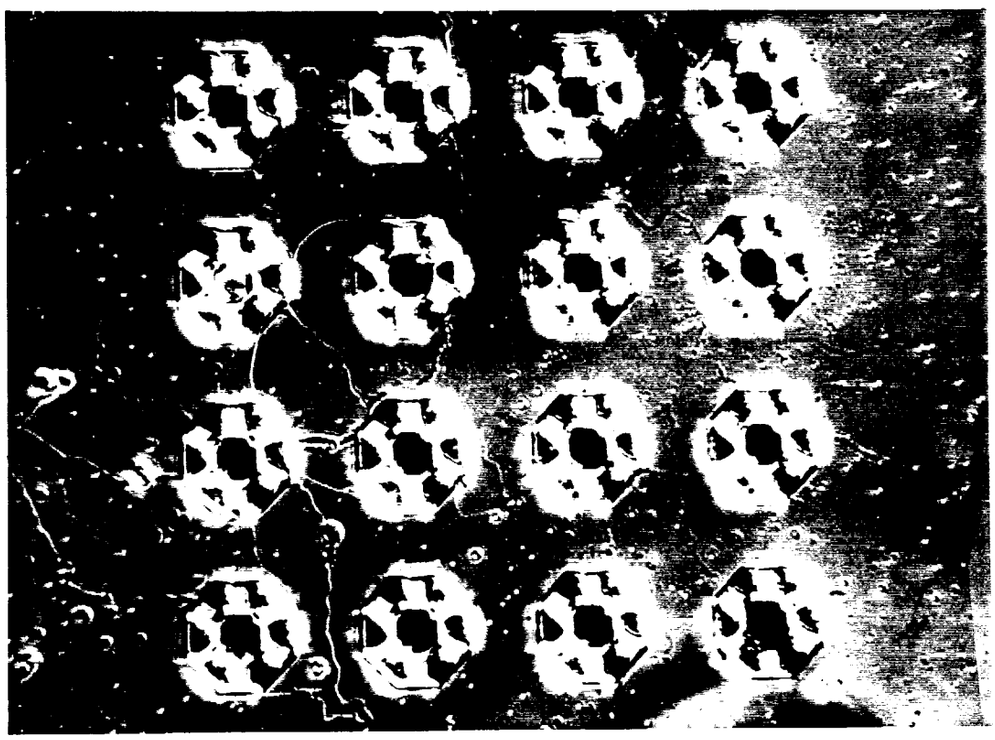
in the wells. Viability, defined as the current cell density divided by the plating density, is the preferred means of determining cell survival since it is not necessary to determine the fate of the dead cells. If cell density is not uniform, viability measurements can be hampered by measurement fluctuations and by cell migration. In the following measurements, viability was determined by making each density count in the same, well-defined area on the chip.

The graph below summarizes the data from eight chips. The x-axis is the viability of normally plated cells growing in the same medium and on the same surface as cells in the wells, and the y-axis indicates the total number of wells containing growing neurons divided by the total number of wells loaded - - the viability of cells in wells. Data from the entire life span of these cultures were included, even as the cultures began to die (these are the data with low viabilities). The average slope of the data is near one, suggesting that being in



a well has no detrimental effect upon a cell. This figure does not include data from the first two days in culture, since the grillwork obscures the early stages of process growth and artificially lowers the apparent well occupancy. There appears to be a ceiling; since well occupancies above 0.8 (13/16 wells) were not observed. Part of this effect may be due to the ever-present possibility of escape through the central hole immediately after loading the wells.

An example of the growth of hippocampal neurons from these chips is shown in the photomicrograph below. This particular chip was coated with poly-DL-lysine and laminin, loaded with E18 rat hippocampal neurons, and cultured in NB/B27 medium. The tunnels are 3000 Å high, 10 μm wide, and 15 μm long. The scale is 2.45 cm = 100 μm. This picture was taken at eight days in culture, and live processes can be seen emerging from eleven of sixteen loaded wells. In particular, note the cell in row 3, column 4. Due to a lithography defect, this position has a grillwork, but no well. The cell placed in the center of this grillwork grew processes through the tunnels and remained trapped, even with no well. This phenomenon has been seen in separate attempts, and no cell escape has been seen. Thus, with the design that has evolved over the past year, hippocampal neurons can finally be very effectively trapped.



Escape

Hippocampal neurons rarely escape through the tunnels in the canopy design. Of the 209 cells surviving in the wells of the twelve chips whose cultures survived more than 6 days, only three escaped through the tunnels. Each escape occured within the first 3 days of culturing, and all occured in 10 μm wide tunnels. The table below summarizes this growth record. In each case, the first number indicates the number of cells which had escaped at 6 DIC (days in culture) while the second number indicates the total number of cells with viable processes at 6 DIC. Thus, it appears that escape through the tunnels only occurs in the first few days after loading, and only in the 10 μm wide tunnels (independent of tunnel height).

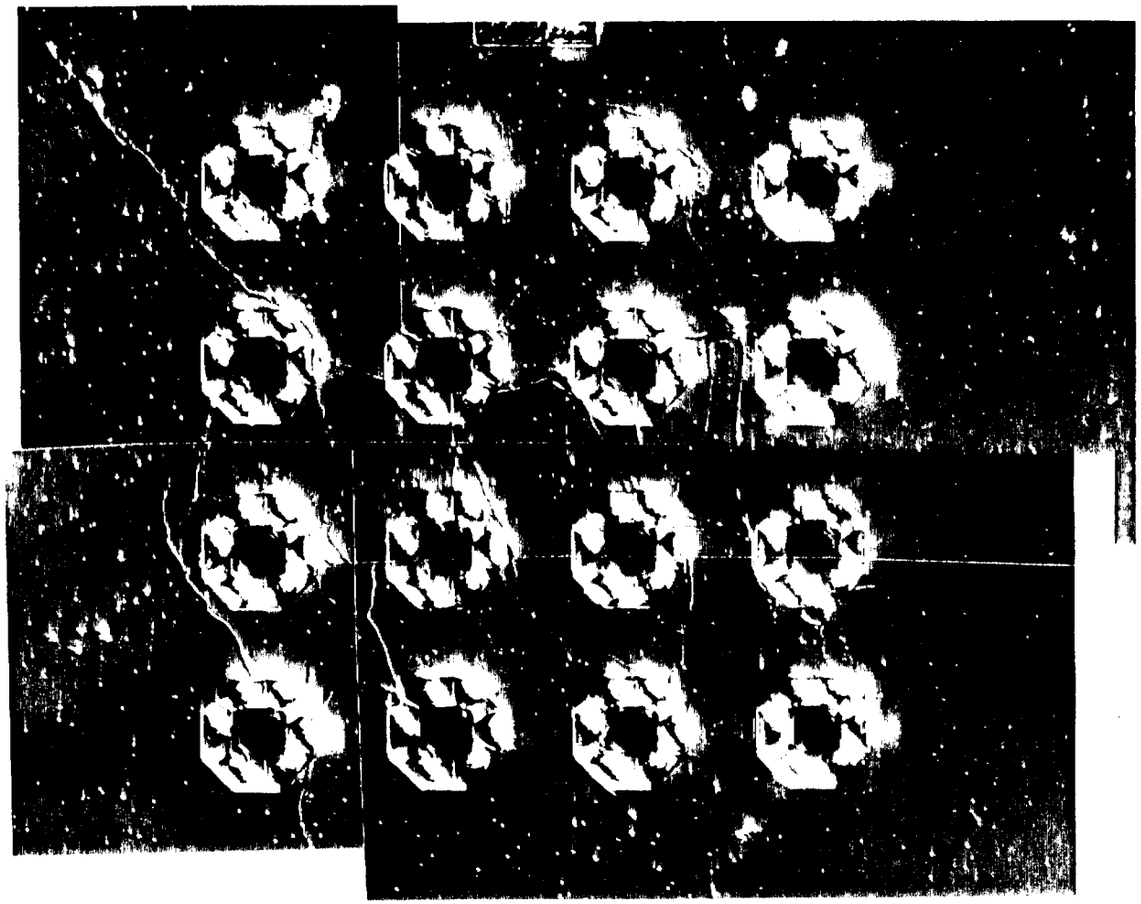
	Tunnel Height		
Tunnel Width	2000 Å	3000 Å	5000 Å
10 μm	2/38	1/33	0/34
4 μm	0/24	0/8	0/18
2 μm	0/20	0/19	0/15

Two of these escapes occured on the same chip, CD14, which had 2000 Å high tunnels. The growth of cells from the 10 μm wide grillwork set are shown in the following photomicrographs, taken at 1, 3, 4, and 6 days in culture. The escapes occured from the wells at row 3, column 3 and row 4, column 2. The cell in (3,3) has a 50 μm long process and a growth cone coming out of the top left tunnel at one day. At three days, the cell has moved 100 μm towards the top, completely escaping the grillwork. A process trails back to the well behind it. In subsequent days, it continues to move and grow. The cell in (4,2) has a 150 μm long, branched process from the top left tunnel at day one, clearly an axon. In the following days, this process continues to grow. At day 3, a small part of the cell body can be seen peeking out of the edge of the tunnel. More of the cell body emerges at day four, and the cell body is completely out of the tunnel at day 6.

CD 14 2000 Å 8/4/95 1 DIC

CD 14 2000 Å 8/6/95 3 DIC

CD 14 2000 Å 8/7/95 4 DIC



CD 14 2000 Å 8/9/95 6 DIC

Conclusions

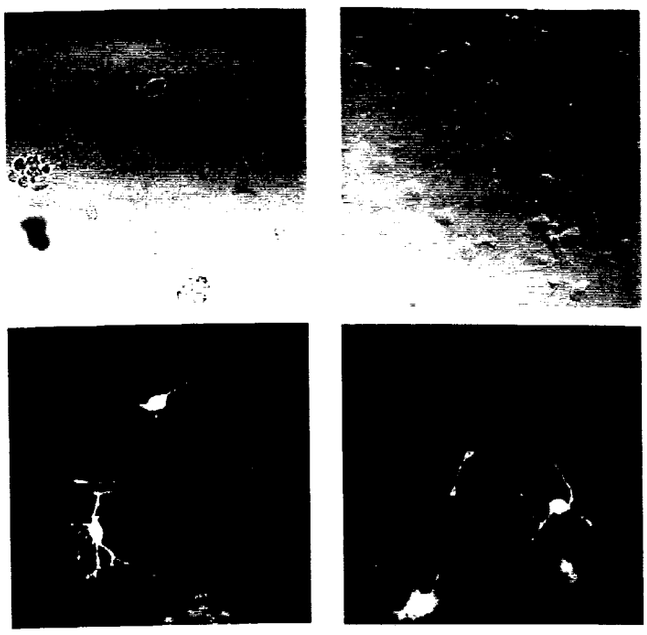
Based on these results, it is clear that the new canopy grillwork design is ideal for hippocampal neurons. Cell survival in the wells is high and the escape rate is extremely low. Cell escape was only observed through the 10 μm wide tunnels and amounts to less than 3% of the cells loaded. Very-long term survival experiments (i.e. over 2 weeks in culture) have not yet been performed, as we have not yet used serum with chips quantitatively. Since all of the tested designs appear to provide adequate cell retention, to provide for the possibility that mature neural processes will have as much room as possible in the tunnels, we plan to use the largest dimensions tested. The Tai group has now begun to fabricate neurochips and dummy probes with canopy grillwork, with 5000 Å high tunnels. Half the wells will have 4 μm wide tunnels, the other half 10 μm wide. The chips will have 15 μm long tunnels, for good visualization of the processes. The probes will have 30 μm long tunnels to provide maximum protection of growing processes during insertion of the probe into a rat.

Labeling with Green Fluorescent Protein

The jellyfish Aequorea Victoria has provided researchers with a valuable tool: A protein, called green fluorescent protein (GFP), that is naturally fluorescent without the need for additional substrates or cofactors. Through molecular-biological techniques, a wide variety of cells have been transfected with the gene for GFP (reviewed in Prasher, 1995 and adjacent articles), allowing them to express the cytosolic protein and become labeled. When excited with blue light, GFP emits green fluorescence. GFP is especially resistant to photobleaching, compared to the dyes used for fluorescence labeling, and does not interfere with normal cellular functioning. But the most important advantage of using GFP instead of dyes, expecially for studies like ours in which neurons will be observed weeks or months after transplantation, is that the label will persist as long as the cell expresses GFP. Even with our optimized dye staining using DiI, we can only hope to image fine neuronal processes a few weeks after implantation of the neuroprobe, because the dye eventually fades or is concentrated in intracellular vacuoles.

Therefore, we have jumped on the GFP bandwagon and begun work to develop a system for transfecting rat hippocampal neurons with GFP. With the molecular biology expertise of the neighboring lab of Prof. Henry Lester, his technician Jennifer Starey, and a visiting undergraduate researcher, Kimberly Riley, we have successfully cloned the gene for GFP into a mammalian expression vector, the adenovirus. The adenovirus has been made safe for lab use (Kremer and Perricaudet,1995) through the deletion of elements crucial for lytic viral reproduction. These elements have been incorporated into the genome of a human kidney cell line, HEK 293, so the GFP gene-bearing virus can be mass-produced in culture by infecting HEK 293 cells. The reproduction-imcompetent viral particles can then be purified and used to infect neurons.

We used a mutant form of GFP, GFP S65T (Heim et al., 1995), that has improved brightness compared to wild-type GFP. Below are some embryonic rat hippocampal neurons infected by our system, expressing the mutant GFP.

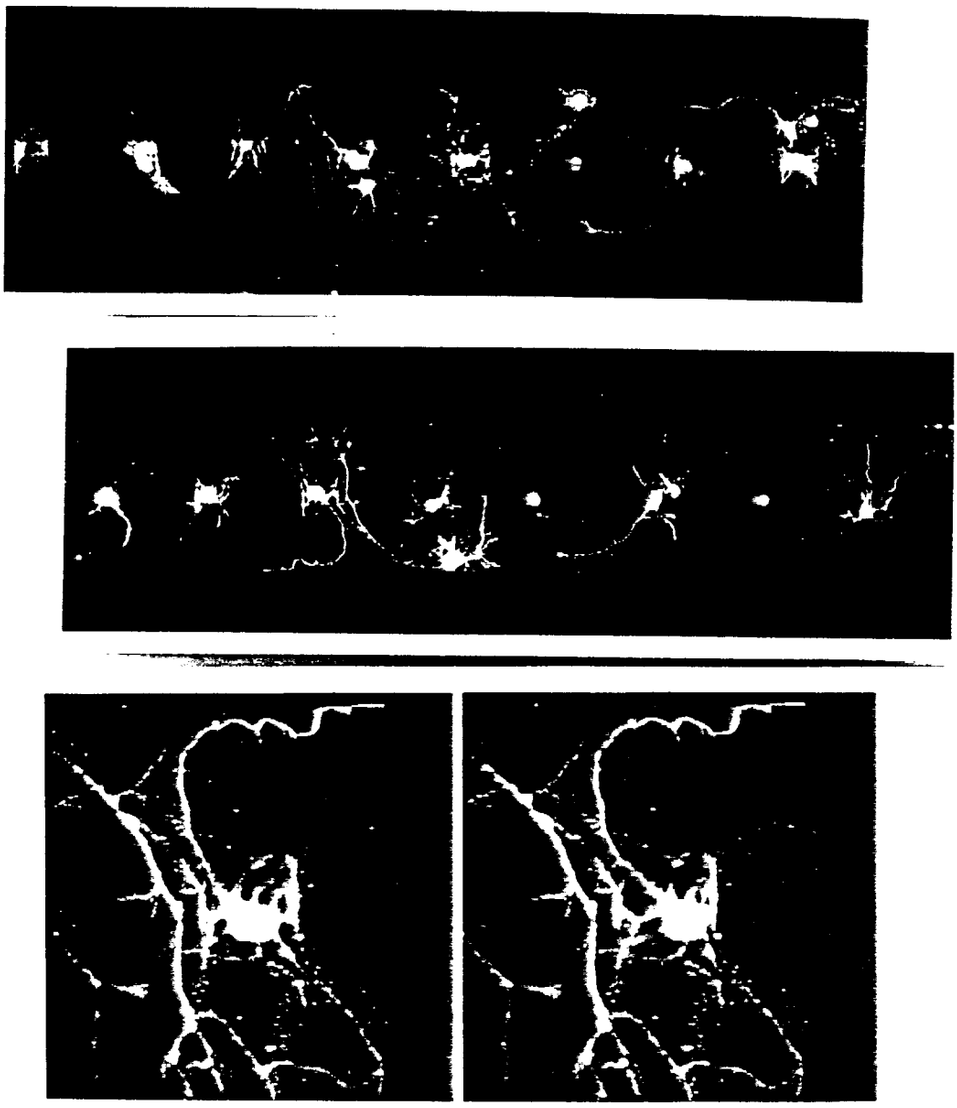


We have demonstrated, for the first time, that GFP is easily detected with 2-Photon excitation at 900 nm, instead of blue or UV excitation (Potter et al, in press) using our 2-photon laser-scanning microscope. No photobleaching or phototoxicity was observed after prolonged imaging of GFP-labeled neurons (even when using a standard mercury arc lamp!). As shown in the picture above, not all neurons express GFP at levels that allow their fine processes to be imaged. We are currently working on enhancing expression levels by adjusting viral titers and improving purity. In the future, we may construct a vector that results in a fusion protein, such as GFP-tubulin or GFP-actin, that would guarantee that the label is present in all of the cell's processes.

Two-Photon time-lapse imaging of hippocampal transplants

We continue to study the *in vitro* system of hippocampal neurons implanted on a cutured hippocampal slice. We continue to improve our cell-staining techniques, which unlike the present GFP labeling system, label 100% of the cells. DiO, a lipophilic dye similar to DiI, is normally excited by blue light, and is substantially brighter than DiI with 2-photon excitation at 900 nm. We have stained embryonic hippocampal neurons with DiO, using our standard DiI

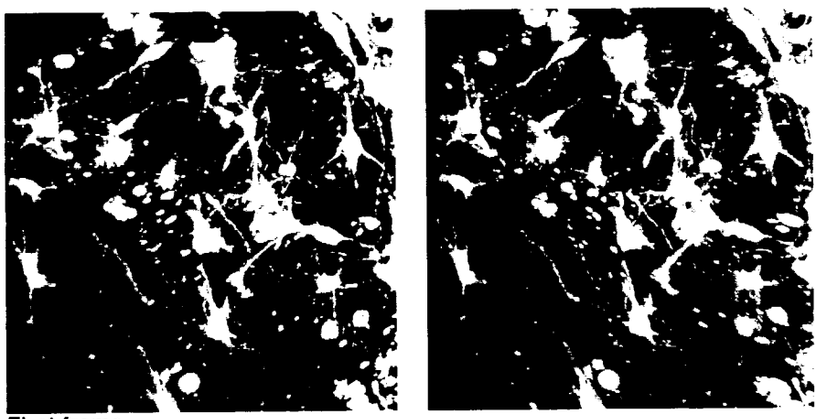
protocol, placed them in neuroprobe wells, and observed excellent survival and outgrowth. In the 2-photon image of a probe shown below, in two halves, at least 11 out of 16 wells had outgrowth after one day in culture. A stereo pair is also shown of a blow-up of well 11, clearly showing the cell body at the bottom of the well, with processes growing up the sides of the well, and out from under the (nearly transparent) grillwork. Non-destructive imaging of living cells with this kind of 3-dimensional detail was not possible before 2-photon microscopy.



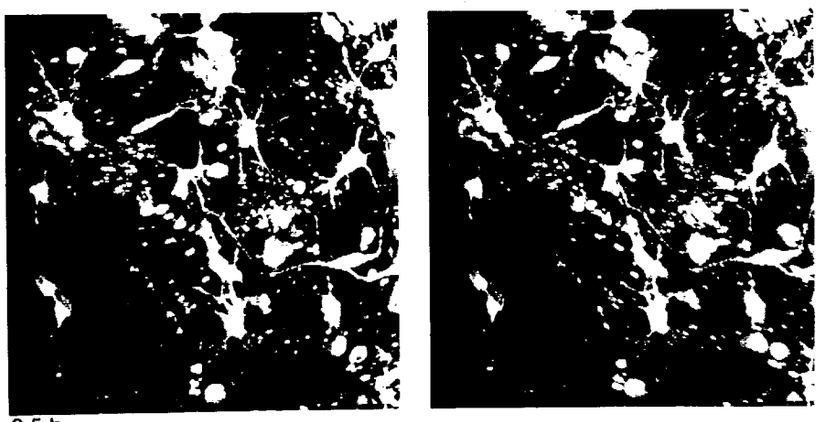
We have placed several neuroprobes showing good outgrowth onto cultured hippocampal slices, and in each case, failed to observed any further outgrowth of probe neurons into the slice. To investigate the possibility that

something about the host slice environment is not permissive to the growth of transplanted neurons, we stained neurons with DiO and plated them directly onto cultured slices, without placing them into neuroprobes. These grow vigorously, sending numerous processes throughout the slice.

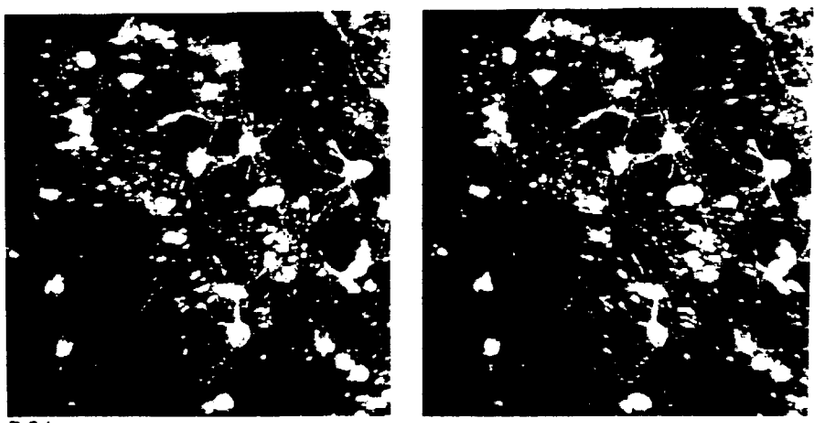
We recently insulated the 2-photon microscope and installed a heating system to allow time-lapse imaging at 37°C. With the enhanced brightness of DiO, it is now possible to image growth cones and fine processes of stained transplants as deep as 150 μm into the slice. Below are three stereo frames excerpted from a time-lapse movie of DiO-stained neurons one day after plating them onto a cultured slice. Each frame of the movie (once every 15 minutes) was made with projections through twenty 2-μm sections (a total of 40 μm into the slice. The field is 250 μm across.) It is clear from the stereo pairs, and even more clear upon viewing the movie, that even the neurons' cell bodies migrate through the entire volume of the slice, extending processes in all three dimensions. They are quite dynamic animals, retracting processes almost as often as they extend them, branching, and sending out growth cones that twist through the unlabeled slice neurons.



First frame



3.5 h



7.5 h

The key points to notice from the stereo pairs are:

• Cells and processes are growing throughout the 40 μm volume that was scanned. There were also cells and processes visible below this volume, down to 150 μm within the slice.

• These are raw, un-enhanced images showing fine processes in excellent detail.

• The labeling is as bright in the last frame as in the first.

• The labeled cells that are alive at the beginning are still growing at the end of the movie. (There is some stained debris, presumably from cells that did not survive the initial dissociation and staining process.)

After 7.5 hours of 2-photon imaging, the cells showed no sign of photobleaching or phototoxicity. This is absolutely unprecedented in the field of confocal microscopy. Photobleaching and phototoxicity limits confocal time-lapse to either a few 3D series images, or a time-series in a single plane. With 2-photon microscopy, we can now do time-lapse in 3 dimensions, limited only by the scanning time of the device, (about 7 sec/scan for the Molecular Dynamics Sarastro 2000 from which our microscope is built), and our disk space. We will soon have a magneto-optical storage device that will allow storage of 3D data for movies lasting several days.

Given that the stained embryonic cells grow well on slices without neuroprobes, we are now investigating each step of the neuroprobe transplantation process to understand the lack of outgrowth from transplanted probes. We hope that the *in vitro* studies will provide information which will help solvce the problem of no outgrowth *in vivo*, which is described below.

References

Heim, R., Cubitt, A. B. and Tsien, R. Y. (1995) Improved green fluorescence. *Nature* **373**: 663-664.

Kremer, E. J. and Perricaudet, M. (1995) Adenovirus and adenoassociated virus-mediated gene-transfer. *British Medical Bulletin* **51**: 31-44.

Potter, S. M., Wang, C.-M., Garrity, P. A. and Fraser, S. E. (In press) Intravital imaging of green fluorescent protein using two-photon laser-scanning microscopy. *Gene*.

Prasher, D. C. (1995) Using gfp to see the light. *Trends In Genetics* **11**: 320-323.

Fabrication

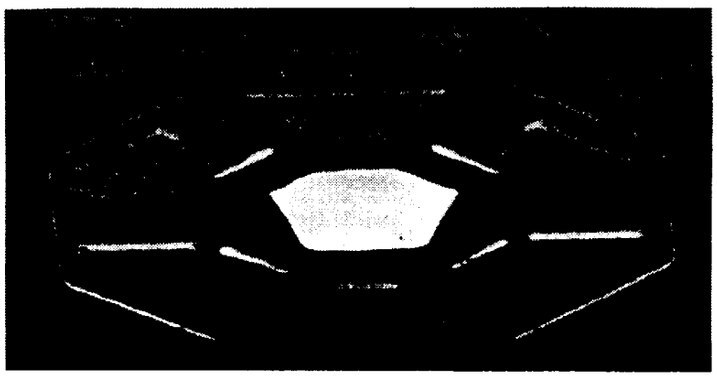
As noted in the last quarterly report, microfabrication was on hold while studies of the dummy neurochips with canopy well designs were being conducted. The aim of the studies was to determine what size channels - length, width and height - were most effective at containing neurons within the wells while still allowing normal outgrowth. The studies showed that neurons seem to grow normally from all of the channel designs investigated. The canopy design turned out to be very effective at keeping neurons from escaping. Only from the 10μm wide channels did any neurons escape during the studies and then, the escape rate was only three percent.

Following the canopy tests, we began to fabricate a batch of dummy neuroprobes for shipment to the Rutgers group. The channel length settled upon is 15μm, the height is 0.5μm. Two channel widths are being fabricated - 4μm and 10μm channels. We have chosen two seperate channel widths to ensure that these probes will be successful. While we showed that neuron growth does not seem to be hindered by the smaller channel size, our studied only lasted two weeks. It may turn out that over longer periods, the finer channels may be to constricting for the neurons. Having a set of larger channels for comparison and for back up is deemed wise at this point.

An additional canopy feature introduced for the probe designs are canopies which extend far beyond the end of the well channels. The Rutgers group has found that the fresh processes extended by the neurons soon after being inserted are broken off when the probe is implanted into the host animal. By fabricating large canopies, the fresh neurites can be sheltered from implantation damage while still allowing researchers to verify neuron outgrowth from the wells before surgery.

We have begun fabrication on a series of neuroprobes and chips using the newly determined canopy dimensions. First to be produced will be dummy neuroprobes which will be sent to Rutgers for implantation work. Second in line will be the production of neurochips with incorporated electrodes. Finally, full-fledged neuroprobes will be fabricated for use in *in vitro* and *in vivo* studies.

Below is an SEM of a canopy well created in the dummy neurochip fabrication run completed last quarter. At the center can be seen the access hole to the neurowell through which the neuron is stuffed. Radiating out from the center are six channels covered with a canopy of nitride. Around these channels are the depressions which act as the support posts for the canopy.



In vivo studies

In the previous quarter we compared the outgrowth of transplanted cell suspensions with the outgrowth of neurons from the wells of probes inserted into the rat's brain. We found outgrowth of axons of transplanted cell suspensions starting one week after transplantation, but we did not find outgrowth from neurons in silicone probes. This could not be a result of the environment during the implanting of neurons in the probes, because neurons on handle areas which touched the brain and then were broken off continued to grow when we put them back into tissue culture. The reason for the absence of outgrowth is not clear. It could be a result of mechanical damage when probes were moved through the host brain during insertion.

We tried to test this hypothesis in our recent experiments. The dummy probes were of the next-to-last design, with no overhang but large corner holes, since the new design was not yet available on probes.

Methods

The preparation of a stained cell suspension and the loading of neurons into wells were the same as in previous experiments. After 24-48 hours of cell culture in the probes, an adult rat was deeply anesthetized and the scalp was opened. The brain meninges were removed by scissors, and the surface of the brain was covered by saline. Probes were them placed into the brain with a stereotaxic holder and left in for periods of ten minutes, one hour, and two hours. Then the probes were removed and placed in a dish with tissue culture medium, and culturing was continued. The examination of outgrowth was done 48 hours later.

Results

Six probes with 100% loading of cells were used for experiments. After 24-48 hours of culture, 52 of the 96 neurons remained in the wells.. Eleven of these showed process outgrowth. Three of the six probes were transplanted into a rat brain for 10 sec, one hour and two hours. The other three were used as controls, and remained in culture. The results are summarized in the table below. It seems as if even a very short time of probe insertion stops neuron

outgrowth, or kills the cells. In these experiments those two alternatives could not be differentiated

Probe Number	Cells in wells	Culture time	Cells after culturing	Cells Growing	Cells in in wells after implant	Growing Cells	Duration of implant
DP-31	16	48	13	2	control	6	0
DP-34	16	48	8	1	control	7	0
DP-37	16	24	12	2	control	5	0
DP-45	16	24	6	4	5	none	10 min
DP-49	16	48	8	2	6	none	1 hr.
DP-53	16	24	5	0	3	none	2 hr

Five of 19 neurons escaped from the wells after implantation, removal, and replacement into the Petry dish. There were no growing cells among the 6 which showed growing processes before the implantation procedure.

The neurons which were on the surface of the silicon probes, with processes and without, were washed out during the transplantation procedure. Examination of the probes 24-48 hours after 2nd culturing showed no processes at all. The surface of the silicon probes was covered by a thin semitransparent film. Part of the cells were visible under the film within the wells, but there were no processes observed.

It is early to draw final conclusions from these results because of the small amount of data, and more additional experiments are required. It is not clear yet whether the film which covered the surface of the silicon probes prevented the growth of cell processes. In future experiments we will use the new type of probes, which protect the proximal ends of neuron processes from damage during theimplantation procedure, and which also prevent escape. We expect better statistical accuracy, and less damage to neurons which are already growing processes when cells are inserted.

In future experiments it will also be determined with a live-dead stain whether neurons after implantation and removal are alive or have been killed. If

death is the result, then it will be necessary to try to determine the cause and to try to control the brain environment near the probe so as to avoid it.